

# Direct Membrane-Damaging Effect of Ketoconazole and Tioconazole on *Candida albicans* Demonstrated by Bioluminescent Assay of ATP

STEFFAN ÅNSÉHN\* AND LENNART NILSSON

Department of Clinical Bacteriology, Linköping University, Regionsjukhuset, S-581 85 Linköping, Sweden

Received 23 November 1983/Accepted 12 April 1984

**Bioluminescent assays of fungal ATP in cultures of *Candida albicans* exposed to tioconazole and ketoconazole demonstrated that intracellular ATP levels were directly related to cell viability. At high concentrations of drug, a fungicidal effect was indicated by a very rapid and heavy leakage of ATP and a simultaneous, dramatic decrease in intracellular ATP. This leakage was due to direct membrane damage, which was less pronounced after exposure to ketoconazole than to tioconazole. After exposure to lower imidazole concentrations, intracellular ATP indicated growth inhibition without significant leakage of ATP. These findings support the hypothesis that imidazole antifungal agents basically act in the same manner but at different concentrations.**

Two modes of action have been suggested for the antimycotic imidazoles clotrimazole and miconazole (10, 11), but not for ketoconazole (11). At low concentrations, all imidazoles have a fungistatic action related to blocked ergosterol synthesis (11). At high concentrations, clotrimazole and miconazole, but not ketoconazole, have a very rapid fungicidal action due to direct membrane damage, which is unrelated to blocked ergosterol synthesis (11). However, conflicting results regarding the membrane-damaging effect of ketoconazole have been presented. For example, high concentrations of miconazole, but not of ketoconazole, have been shown to cause a rapid  $K^+$  release from *Candida* species (2); on the other hand, high concentrations of ketoconazole have been shown to cause leakage of nucleic acids and proteins from *Candida albicans* (13).

The antifungal activity of antimycotics can be assayed by using a luciferase assay of ATP to determine viable fungal biomass (8). We have previously shown that, in cultures of *C. albicans* exposed to econazole, intracellular ATP levels are directly related to viable-cell counts (1) and that agents causing damage to bacterial membranes cause a leakage of ATP into the medium. This leakage interferes with the determination of intracellular ATP; thus, procedures have been developed that selectively determine intracellular and extracellular ATP (7; L. Nilsson, Ph.D. dissertation, Linköping University, Regionsjukhuset, Sweden, 1981). In the present report, we have studied extracellular and intracellular ATP in *C. albicans* exposed to tioconazole and ketoconazole. The results were correlated with cell viability to further elucidate the mode of action of these drugs.

## MATERIALS AND METHODS

**Antifungal agents.** Ketoconazole (Leo AB, Helsingborg, Sweden) and tioconazole (Pfizer, Inc., Sandwich, England) were each dissolved in 100% dimethyl sulfoxide (DMSO) to stock solutions of 10 mg/ml. Just before each experiment, working solutions were prepared in the medium to be used.

**Test organisms.** *C. albicans* Y01/07 (Pfizer, Inc.) and *C. albicans* H-29 (Hoffmann-La Roche, Inc., Basel, Switzerland) were maintained on Sabouraud agar (Oxoid Ltd., London, England).

**Test media and preparation of inocula.** The test organisms

were incubated at 37°C for 18 to 24 h in yeast nitrogen base (YNB) broth (Difco Laboratories, Detroit, Mich.) supplemented with glucose and L-asparagine (9). Dilutions of these overnight cultures were prepared in test medium or buffer immediately before the start of an experiment. Cells were exposed to imidazole antifungal agents in one of the following media: unbuffered YNB; YNB buffered to pH 7.0 with citrate phosphate ( $\text{NaHPO}_4 \cdot 2\text{H}_2\text{O}$ ) (1.936 g/liter) and citric acid (1.220 g/liter; 0.0165 M), with Britton Robinson (BR; citric acid [6.935 g/liter],  $\text{KH}_2\text{PO}_4$  [4.491 g/liter], boric acid [2.040 g/liter], barbitol [6.079 g/liter], or with MOPS (3-[morpholino]propanosulfonic acid-sodium) (0.1 M); or one of the above buffers without YNB.

**Viability.** Viable-cell count was determined (CFU per milliliter) by plating in duplicate on Sabouraud agar after the appropriate serial dilution.

**Luciferase assay of fungal ATP.** (i) **Analytical equipment.** Light emission from the bioluminescent assay was measured in a 1250 luminometer (LKB-Wallac, Turku, Finland) and recorded on a 2210 potentiometric recorder (LKB-Products, Bromma, Sweden). The heat extraction of intracellular fungal ATP was performed in an LKB-Biocal 2973 incubator (LKB-Products).

(ii) **Analytical reagents.** ATP-monitoring reagent (LKB-Wallac) was used in the assay of ATP. Apyrase (purified grade I) (Sigma Chemical Co., St. Louis, Mo.) was used to eliminate extracellular ATP before the extraction of intracellular ATP. Other reagents were of analytical grade.

**Intracellular ATP levels in cultures of *C. albicans*.** Twofold serial dilutions of ketoconazole and tioconazole were prepared in appropriate test media (0.9 ml) and inoculated with a 10-fold dilution of the overnight culture (0.1 ml) giving a final yeast concentration of ca.  $10^6$  CFU/ml. Each experiment included a control culture that was not exposed to antifungal agents. The yeast cultures were incubated at 37°C with shaking, and intracellular ATP was determined by the following procedure.

(i) **Elimination of extracellular ATP.** A 50- $\mu$ l sample from the culture was incubated for 10 min at 37°C with 50  $\mu$ l of an apyrase solution consisting of 0.04% apyrase made up in YNB.

(ii) **Extraction of intracellular ATP.** After elimination of extracellular ATP, 50  $\mu$ l of the apyrase-treated sample was pipetted into 450  $\mu$ l of boiling 0.1 M Tris buffer, pH 7.75, containing 2 mM EDTA. After heating for 90 s, the extracts

\* Corresponding author.

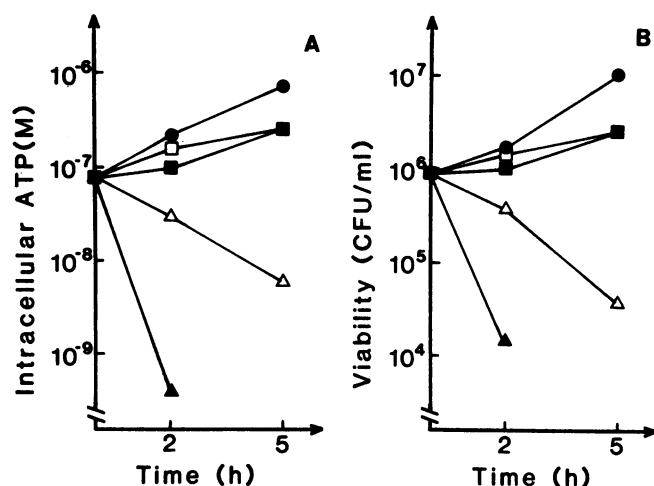


FIG. 1. Relationship between intracellular ATP (A) and viability (CFU/ml) (B) in *C. albicans* H-29 cultures exposed to tioconazole in the following concentrations ( $\mu\text{g/ml}$ ): 125 ( $\blacktriangle$ ), 62.5 ( $\triangle$ ), 31.25 ( $\blacksquare$ ), and 15.75 ( $\square$ ).  $\bullet$ , Control without antifungal agent. The experiment was performed in unbuffered YNB broth.

were cooled before the assay of ATP. Extraction inhibited the action of the apyrase and disrupted the yeast cells, thus releasing their ATP content.

(iii) **Luciferase assay of ATP.** Luciferase reagent (100  $\mu\text{l}$ ) was added to 500  $\mu\text{l}$  of each extract, and the light intensity was recorded.

(iv) **Calculation of assay results.** Sample ATP levels were calculated by using assays of standard amounts of ATP as references, and correction for background luminescence was made. Known amounts of ATP added to the extracts were

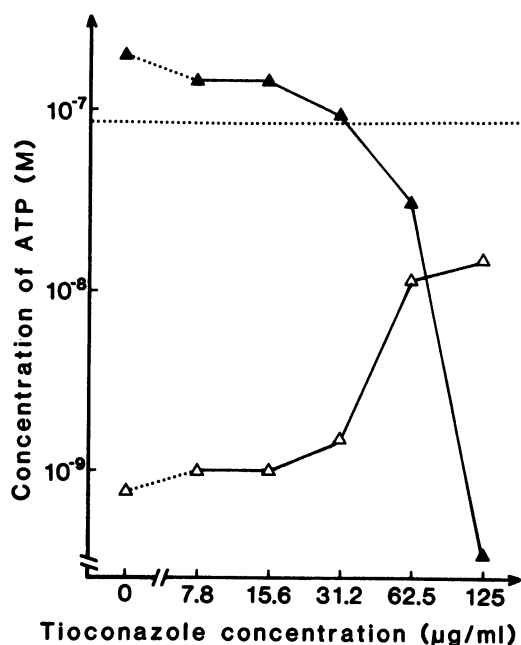


FIG. 2. Relationship between intracellular ( $\blacktriangle$ ) and extracellular ( $\triangle$ ) ATP in *C. albicans* H-29 cultures exposed to tioconazole for 2 h. The experiment was performed in unbuffered YNB broth. The dotted line indicates the intracellular ATP level at the start of the experiment.

TABLE 1. Viability and methylene blue uptake by *C. albicans* in cultures exposed to imidazoles for 18 h<sup>a</sup>

Imidazole concn [ $\mu\text{g/ml}$ (mM)]	Viability (CFU/ml) in:		% Stained cells in:	
	YNB	YNB- MOPS	YNB	YNB- MOPS
<b>Tioconazole</b>				
0 <sup>b</sup> (0)	$6.8 \times 10^7$	$1.7 \times 10^7$	0	0
0 (0)	$6.8 \times 10^7$	$6.6 \times 10^7$	0	0
50 (129)	$3.0 \times 10^6$	$2.0 \times 10^4$	40	62
100 (258)	$4.0 \times 10^3$	0 <sup>c</sup>	96	100
200 (516)	0	0	100	100
400 (1,032)	0	0	100	100
<b>Ketoconazole</b>				
50 (95)	$7.2 \times 10^6$	$7.0 \times 10^6$	9	6
100 (190)	$5.6 \times 10^6$	$2.5 \times 10^6$	14	20
200 (380)	$2.0 \times 10^6$	$1.5 \times 10^5$	46	48
400 (760)	0	0	48	50

<sup>a</sup> pH (YNB), 5.6; pH (YNB-MOPS), 7.0.

<sup>b</sup> Control containing 4% DMSO.

<sup>c</sup> 0, Below the detection limit of 10 CFU/ml.

used as internal standards to correct for inhibition of the luciferase reaction by the extracts.

**Extracellular ATP levels in cultures of *C. albicans*.** To get a constant light when assaying extracellular ATP in yeast cultures, we buffered the commercially available luciferase reagent with 0.2 M Tris, pH 7.75, containing 4 mM EDTA. The buffered luciferase reagent (250  $\mu\text{l}$ ) was added directly to the yeast cultures (250  $\mu\text{l}$ ), and the light intensity, which was constant for several minutes, was recorded. All assays were performed in duplicate. Assay results were calculated as described above.

**Kinetics of ATP leakage from *C. albicans* exposed to ketoconazole and tioconazole.** A portion (200  $\mu\text{l}$ ) of luciferase reagent was added to each of a series of test tubes containing 0.9 ml of ketoconazole or tioconazole solution in buffer per tube, and then 0.1 ml of an overnight culture of *C. albicans* was added. The light intensity was recorded at short time intervals (minutes). Each experiment included, as controls, *C. albicans* isolates that had not been exposed to antifungal agents and known amounts of ATP in test medium. These experiments were performed at 22°C. The calculation of assay results was performed as described above.

**Methylene blue uptake.** The direct membrane-damaging effect of the imidazoles was tested by methylene blue uptake as described by Sud and Feingold (10).

## RESULTS

**Relationship between intracellular ATP and cell viability in *C. albicans* cultures exposed to tioconazole.** In tioconazole-exposed cultures of *C. albicans* H-29, intracellular ATP was compared with cell viability (CFU per milliliter) (Fig. 1). Tioconazole gave a dose-dependent reduction of growth and exerted a fungicidal effect at 62.5 and 125  $\mu\text{g/ml}$ . At these concentrations, cell viability was reduced rapidly, and at 125  $\mu\text{g/ml}$ , no viable cells were found and the intracellular ATP level was below the detection limit ( $10^{-10}$  M) after 5 h of incubation. Corresponding concentrations of ketoconazole did not affect viability and intracellular ATP under the same experimental conditions.

**Relationship between intracellular and extracellular ATP in *C. albicans* cultures exposed to tioconazole.** A growth inhibition in *C. albicans* H-29 cultures exposed to tioconazole occurred at 7.8 to 31.2  $\mu\text{g/ml}$  (Fig. 2). At 62.5  $\mu\text{g/ml}$  of tioconazole per ml, the intracellular ATP decreased to below

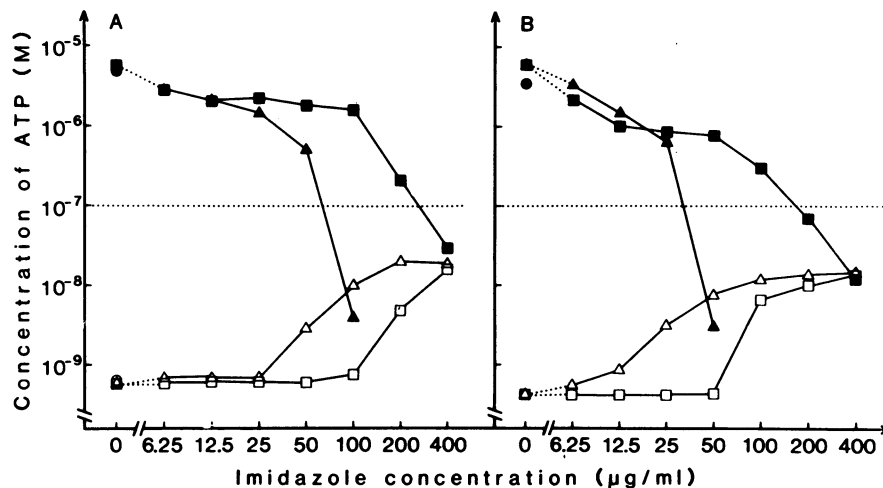


FIG. 3. Effect of the antifungal activity of ketoconazole ( $\square$ ,  $\blacksquare$ ) and tioconazole ( $\triangle$ ,  $\blacktriangle$ ) on *C. albicans* Y01/07 cultures. The experiments were performed (A) in unbuffered YNB broth, pH 5.6, and (B) in YNB broth buffered with MOPS, pH 7.0. Intracellular ATP (closed symbols) was determined after 18 h of incubation. Extracellular ATP (open symbols) was determined after 2 h of incubation. The dotted line indicates the intracellular ATP level at the start of the experiment.  $\circ$ ,  $\bullet$ , Control without antifungal agent but containing 4% DMSO.

the starting level concomitantly with a dramatic increase of extracellular ATP. This indicates a correlation between ATP leakage and decreased cell viability.

**Comparison of the antifungal effects of ketoconazole and tioconazole on ATP levels in *C. albicans* cultures.** The intracellular ATP levels in *C. albicans* Y01/07 cultures increased 60-fold after 18 h of incubation (Fig. 3). The high imidazole concentrations tested in this experiment necessitated the use of DMSO at a final concentration of 4%. This concentration of DMSO had little or no growth-inhibiting effect as demonstrated by cell viability and intracellular ATP (Fig. 3 and Table 1). In cultures exposed to  $\leq 200$   $\mu\text{g}$  of ketoconazole per ml, growth inhibition occurred (Fig. 3A). At 400  $\mu\text{g}$  of ketoconazole per ml, the intracellular ATP levels and cell viability decreased to below the starting level, indicating a fungicidal effect (Fig. 3A and Table 1).

A dramatic increase in extracellular ATP was demonstrated at 200 and 400  $\mu\text{g}$  of ketoconazole per ml. The extracellular ATP levels in control cultures with and without DMSO (4%) were equal.

Tioconazole exerted a partial growth inhibition at  $\leq 50$   $\mu\text{g}/\text{ml}$  and a fungicidal effect at  $\geq 100$   $\mu\text{g}/\text{ml}$  (Fig. 3A and Table 1).

Membrane damage as demonstrated by methylene blue uptake (10) was tested. At high concentrations of ketoconazole (200 and 400  $\mu\text{g}/\text{ml}$ ), ca. 50% of the cells were stained, but in cultures exposed to 100, 200, and 400  $\mu\text{g}$  of tioconazole per ml, 100% of the cells were stained. At lower concentrations, fewer cells were stained, and in control cultures with and without 4% DMSO, no cells were stained (Table 1).

*C. albicans* decreased the pH of unbuffered YNB from 5.6 to 3.0 within 4 h of growth. If YNB was buffered with 0.1 M MOPS (pH 7.0), the pH was not significantly changed after 24 h of growth. Both ketoconazole and tioconazole had more pronounced antifungal activities in YNB buffered with MOPS than in unbuffered YNB (Fig. 3B and Table 1).

**Kinetics of the ATP leakage from *C. albicans* exposed to ketoconazole and tioconazole.** The leakage of ATP from *C. albicans* Y01/07 exposed to ketoconazole or tioconazole at

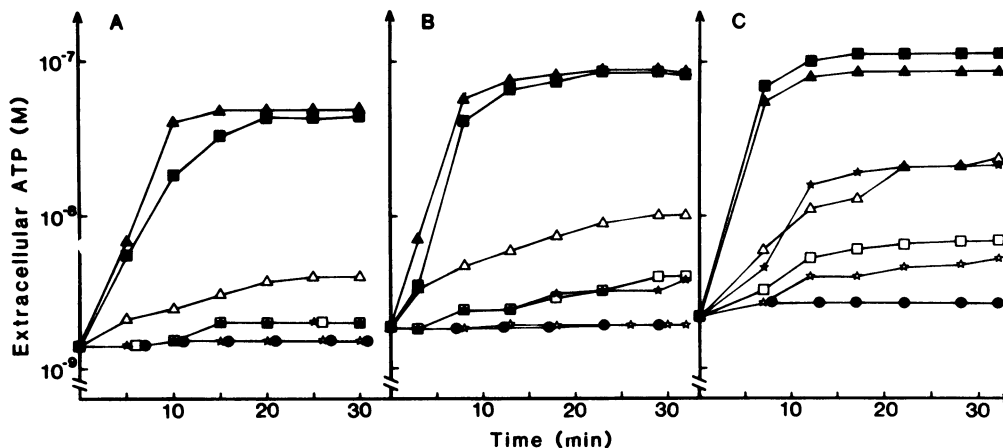


FIG. 4. Kinetics of ATP leakage from *C. albicans* Y01/07 in MOPS (A), citrate phosphate (B), and BR (C) buffer, pH 7.0, when exposed to concentrations ( $\mu\text{g}/\text{ml}$ ) of ketoconazole (75 [ $\triangle$ ], 37.5 [ $\square$ ], and 18.75 [ $\star$ ]) and of tioconazole (75 [ $\blacktriangle$ ], 37.5 [ $\blacksquare$ ], and 18.75 [ $\blackstar$ ]).  $\bullet$ , Control in appropriate buffer without antifungal agent.

22°C in different buffers is shown in Fig. 4. High concentrations of tioconazole (37.5 and 75 µg/ml) caused very rapid and extensive leakage, with maximum ATP levels reached within 15 min of exposure. The magnitude of the leakage was the same in the different buffers. At a lower tioconazole concentration (18.75 µg/ml), the leakage was more pronounced in BR than in citrate phosphate and MOPS buffers. The magnitude of ATP leakage caused by ketoconazole was less than that caused by tioconazole.

In cultures not exposed to imidazoles and in those without leakage of ATP, less than 5% of the *C. albicans* cells were stained by methylene blue. At high concentrations of tioconazole (37.5 and 75 µg/ml), >50% of the cells were stained after 5 min of exposure, and the number increased to ≥90% within 20 min. A high concentration of ketoconazole (75 µg/ml) caused ≤10% of the cells to become stained within 10 min in BR buffer, and the number increased with time to 50%.

### DISCUSSION

In a study of interactions among antimycotics, the levels of ATP in fungal cultures were found to correlate with the amounts of viable fungal biomass (8). However, in cultures exposed to subinhibitory concentrations of amphotericin B, increased cell membrane permeability gave higher ATP levels than those in drug-free control cultures (8). These higher ATP levels might have been due to the accumulation of extracellular ATP. Thus, it is necessary to selectively determine intra- and extracellular ATP when membrane-active agents are investigated. In this study, extracellular ATP was eliminated by apyrase before the extraction and assay of intracellular ATP, and extracellular ATP was determined by adding luciferase reagent to untreated yeast cultures.

The results in this and in a previous study (1) show that intracellular ATP levels are directly related to cell viability in cultures of *C. albicans* exposed to imidazole antifungal agents. Tioconazole caused a dose-dependent decrease in intracellular ATP in growing *C. albicans* cultures. Concentrations of the drug high enough to cause growth inhibition gave intracellular ATP levels between the starting level and the level of the unexposed control. At these concentrations, little or no accumulation of extracellular ATP was found. Higher tioconazole concentrations resulted in intracellular ATP levels below the starting level and a concomitant accumulation of extracellular ATP. The tioconazole-induced membrane damage resulted in ATP leakage which was dose dependent up to a maximum level at which all cells were stained by methylene blue and cell viability was lost. A further increase in the tioconazole concentration did not raise the extracellular ATP level. In kinetic studies, maximum extracellular ATP levels were reached within 10 to 15 min of exposure to high concentrations of tioconazole. This rapid leakage, which does not require growth, supports the concept of Sud and Feingold that some imidazole antifungal agents are fungicidal at high concentrations as a result of a direct membrane damage (10, 11).

In the present study, we observed that ketoconazole exerted antifungal activities that were similar to those of tioconazole but only at higher concentrations of ketoconazole. The antifungal activity of ketoconazole was better in buffered (pH 7.0) than unbuffered (pH 5.6) YNB broth, which is consistent with other observations (6). The ATP leakage induced by the imidazoles, and especially by ketoconazole, varied with the kind of buffer used. Differences in buffer composition might explain the differences in ATP

leakage, since the addition of even very small amounts of divalent cations has been shown to inhibit leakage of extracellular constituents from *C. albicans* caused by miconazole (12). Thus, test conditions greatly influence the in vitro activities of imidazole antifungal agents, which is a well-known phenomenon (3–6, 12).

When the antifungal activities of imidazole antifungal agents are assessed, the possibility must be considered that the solvents used might themselves have some antifungal activity. The highest imidazole antifungal concentration tested in this report required DMSO at a final concentration of 4%. This concentration of DMSO had, in itself, little or no growth-inhibiting effect as indicated by cell viability and the intracellular ATP level. However, no membrane damage was demonstrated by leakage of ATP or by methylene blue uptake.

The present study shows, in contrast to that by Sud and Feingold (11) and by Beggs (2), but in accordance with the study by Uno et al. (13), that ketoconazole can exert a direct membrane-damaging effect on yeast cells. These findings support the hypothesis (13) that imidazole antifungal agents basically act in the same manner but at different concentrations.

Furthermore, the bioluminescent assay of ATP might also indicate two modes of action, as previously suggested (10, 11). At low concentrations, intracellular ATP levels indicated growth inhibition, whereas at high concentrations, a fungicidal effect was indicated by a very rapid and extensive leakage of ATP with a concomitant, dramatic decrease in intracellular ATP.

### LITERATURE CITED

1. Ånséhn, S. 1977. In vitro synergistic action of antimycotics and antibiotics on *Candida albicans*. *Curr. Ther. Res. Clin. Exp.* 22:92–99.
2. Beggs, W. H. 1983. Comparison of miconazole- and ketoconazole-induced K<sup>+</sup> release from *Candida* species. *J. Antimicrob. Chemother.* 11:381–383.
3. Borgers, M. 1981. Pharmakologie und Mikrobiologie der Imidazolderivate. *Mykosen* 24:59–69.
4. Haller, I. 1979. Modern aspects of testing azole antifungals. *Postgrad. Med. J.* 55:681–682.
5. Hoepferich, P. D., and A. C. Huston. 1976. Effect of culture media on the antifungal activity of miconazole and amphotericin B methyl ester. *J. Infect. Dis.* 134:336–341.
6. Minagawa, H., K. Kitaura, and N. Nakamizo. 1983. Effects of pH on the activity of ketoconazole against *Candida albicans*. *Antimicrob. Agents Chemother.* 23:105–107.
7. Nilsson, L. 1978. New rapid bioassay of gentamicin based on luciferase assay of extracellular ATP in bacterial cultures. *Antimicrob. Agents Chemother.* 14:812–816.
8. Odds, F. C. 1982. Interactions among amphotericin B, 5-fluorocytosine, ketoconazole, and miconazole against pathogenic fungi in vitro. *Antimicrob. Agents Chemother.* 22:763–770.
9. Shadomy, S. 1969. In vitro studies with 5-fluorocytosine. *Appl. Microbiol.* 17:871–877.
10. Sud, I. J., and D. S. Feingold. 1981. Mechanisms of action of the antimycotic imidazoles. *J. Invest. Dermatol.* 76:438–441.
11. Sud, I. J., and D. S. Feingold. 1981. Heterogeneity of action mechanisms among antimycotic imidazoles. *Antimicrob. Agents Chemother.* 20:71–74.
12. Swamy, K. H. S., M. Sirsi, and G. R. Rao. 1974. Studies on the mechanism of action of miconazole: effect of miconazole on respiration and cell permeability of *Candida albicans*. *Antimicrob. Agents Chemother.* 5:420–425.
13. Uno, J., M. L. Shigematsu, and T. Arai. 1982. Primary site of action of ketoconazole on *Candida albicans*. *Antimicrob. Agents Chemother.* 21:912–918.